Detection of imipenem-resistant and metronidazole-resistant *Bacteroides fragilis* group strains in fecal samples

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Objective: To investigate the imipenem and metronidazole resistance profiles of *Bacteroides fragilis* group strains in fecal samples and to detect the resistance genes (*ccrA* and *nim*) coding for imipenem and metronidazole resistance in *B. fragilis* group strains.

Methods: In total, 925 fecal samples, 729 from consecutive diarrhea patients and 196 from healthy controls, were collected at Huddinge University Hospital in 1997. A modified disk diffusion method was employed to screen for imipenem-resistant and metronidazole-resistant *B. fragilis* group strains. In strains considered resistant by the modified disk diffusion method, the minimum inhibitory concentrations (MICs) were further determined by the agar dilution method. PCR assays were used to detect the carbapenem-hydrolyzing metallo-β-lactamase gene (*ccrA*) and the 5-nitroimidazole resistance genes (*nim*) in pure cultures (purePCR), directly from fecal samples through direct broth enrichment (dirPCR) and by immunomagnetic separation (imsPCR).

Results: Two imipenem-resistant *B. fragilis* strains, one of which was simultaneously resistant to metronidazole, and two *B. fragilis* group strains with MICs near the breakpoint for metronidazole resistance, were isolated from the fecal samples of diarrhea patients. The *ccrA* gene was identified in all the imipenem-resistant *B. fragilis* strains by purePCR, dirPCR and imsPCR. The *nim* genes were also detectable by these PCR assays.

Conclusions: The incidences of imipenem-resistant and metronidazole-resistant *B. fragilis* group strains were low in the investigated diarrhea patients. Simultaneous resistance to imipenem and metronidazole is of great concern in clinical medicine, and the proposed PCR assays may be useful in epidemiologic studies of distribution of resistance genes in the fecal microflora.

Key words: *Bacteroides fragilis* group, imipenem, metronidazole, resistance, purePCR, dirPCR, imsPCR

INTRODUCTION

Bacteroides fragilis group strains constitute the major part of the intestinal microflora [1]. They are the micro-organisms most often isolated from patients with anaerobic infections and among the most resistant of all anaerobes to antimicrobial agents [2,3]. Imipenem and metronidazole are two of the most active antimicrobial agents against the *B. fragilis* group [3–6]. Although rare, resistance to imipenem or metronidazole among clinical isolates of the *B. fragilis* group exists [3,5, 7–9,10].

The primary mechanism for resistance to β-lactam agents is production of β-lactamases. Carbapenem-hydrolyzing metallo-β-lactamase plays an important role in the imipenem resistance of *B. fragilis* strains. This enzyme production is encoded by *ccrA* (or *cfiA*) [3,11]. A PCR technique has been established to detect the metallo-β-lactamase gene (*ccrA*) in pure cultures of *B. fragilis* strains [12]. Stool samples are some of the most difficult specimens on which to perform PCR, due to the presence of multiple substances that inhibit the activity of polymerase [13,14]. Recently, in the case of enterotoxigenic *B. fragilis* (ETBF), a culture method was used by Pantosti et al to prepare fecal samples for PCR analysis [15], and an imsPCR assay, based on immunomagnetic separation (IMS) in combination with PCR, was developed by Zhang and Weintraub [16] to detect the enterotoxin gene.

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5-Nitroimidazole (metronidazole) resistance appears to be caused by a combination of decreased antibiotic uptake and decreased nitroreductase activity [3]. It has been demonstrated that the *nim* genes confer a moderate level of resistance to metronidazole on *Bacteroides* spp. strains [9,17]. A PCR method has been developed by Trinh and Reysset for detection of the *nim* genes (*nimA*, *nimB*, *nimC* and *nimD*) encoding 5-nitroimidazole resistance in *Bacteroides* spp. The expected fragment was obtained by PCR from *Bacteroides* strains with MICs of 4–32 mg/L [18].

In the present study, we investigated the imipenem and metronidazole resistance profiles of *B. fragilis* group strains in fecal samples and described the use of PCR to detect the metallo-β-lactamase gene (*ccrA*) and the 5-nitroimidazole resistance genes (*nim*) in pure cultures (purePCR), directly from fecal samples through direct broth enrichment (dirPCR) and by IMS.

MATERIALS AND METHODS

Fecal samples

Fecal samples from 729 consecutive diarrhea patients and 196 healthy controls were collected at Huddinge University Hospital in 1997.

Selective media and culture conditions

The collected fecal samples were frozen at −70°C until use. One cotton swab of thawed specimen was suspended in 1 mL of nutrient broth. Four drops (~200 μL) of the fecal solutions were inoculated in 4.5 mL of prereduced peptone yeast glucose (PYG) broth containing kanamycin (800 mg/L) and incubated at 37°C for 48 h. After incubation, the samples were processed by a modified disk diffusion method. A swab of sample from the enrichment broth was streaked onto blood–kanamycin–vancomycin (BKV; kanamycin 100 mg/L, vancomycin 7.5 mg/L) plates. Imipenem and metronidazole disks (10 μg/disk; AB Biodisk, Solna, Sweden) were applied on the plates, which were incubated anaerobically (BBL GasPak Anaerobic System, Cockeysville, MD, USA) at 37°C for 48 h. Colonies resistant to imipenem (R: diameter <13 mm) or metronidazole (R: no inhibition zone) were subcultured on blood agar plates and identified by Gram-staining, gas–liquid chromatography (GLC) and fermentation tests [19].

Antimicrobial susceptibility tests

The minimum inhibitory concentrations (MICs) of imipenem and metronidazole were determined by the agar dilution method on ASM agar (AB Biodisk, Solna, Sweden) with addition of 5% defibrinated horse blood. The inoculum was approximately 10^5 CFU/spot, applied by a Steer's replicator, and the strains were incubated for 48 h at 37°C in anaerobic jars (BBL GasPak Anaerobic System, Cockeysville, MD, USA) [19]. The agar dilution method employed was generally in accordance with the National Committee for Clinical Laboratory Standards (NCCLS) [20], except that ASM blood agar instead of Brucella blood agar was used. Breakpoints used for imipenem (S ≤4 mg/L, R ≥16 mg/L) and for metronidazole (S ≤8 mg/L, R ≥32 mg/L) were according to the NCCLS [20].

Detection of *ccrA* and *nim* genes by PCR

DNA extraction

1. DNA extraction from pure bacterial colonies (purePCR). One loop (1–2 μL) of bacteria was picked from an agar plate, suspended in 100 μL of MilliQ water, and heated at 95°C for 15 min. Following centrifugation (10 000*g* × 5 min) to remove cell debris, the supernatant containing the DNA was stored at −20°C until use.
2. DNA extraction directly from PYG broth (dirPCR). The fecal samples were processed as described above. The PYG broth (~4.7 mL) was then centrifuged at 600 rev/min for 10 min to remove fecal debris. The supernatant was further centrifuged at 4000 rev/min for another 10 min. The pellet was suspended in 120 μL of MilliQ water. The following procedures were the same as in step 1.
3. DNA extraction from PYG broth by use of IMS (imsPCR). Following two-step centrifugation as described above, the pellet was suspended in 80 μL of phosphate-buffered saline (PBS) for incubation with magnetic beads. IMS was done according to the method of Zhang and Weintraub [16], with minor modifications. The magnetic beads, Dynabeads M-450 and Dynabeads M-280 (Dynal, Oslo, Norway), were washed according to the manufacturer's recommendations. Then, 18 μg of MAb C3 [16] was mixed with 375 μL of Dynabeads M-450, and 30 μg of MAb 4H8 [16] was mixed with 375 μL of Dynabeads M-280. The volumes were adjusted to 4000 μL by PBS with 0.1% bovine serum albumin (BSA), and the beads were incubated overnight with bidirectional rotation at 4°C. The coated beads were washed by PBS containing 0.05% Tween-20 (Merck, Schuchardt, Munich, Germany), and were resuspended in 2250 μL of PBS with 0.1% BSA. Seventy-five microliters each of the above coated beads solution was added to each well of a 96-well microtiter plate (Techne, Cambridge, UK). With the aid of the magnetic separator (Beadprep 96; Techne), the magnetic beads were incubated with the added sample. The beads with bound

bacteria were suspended in 100 µL of MilliQ water and processed as in step 1 to extract the DNA.

Amplification of ccrA

Primers P1 (5'-AAA GAA TAA AAT GAA AAC AGT-3') and P2 (5'-CAG TGA ATC GGT GAA TC-3') directed to the *ccrA* gene were used [12]. The same amplification parameters as described previously were employed, except that varying amounts of DNA from steps 1, 2 and 3 were added in a final volume of 25 µL of PCR mixture. The expected product was a DNA fragment with a length of 442 bp.

Amplification of nim

Primers NIM3 (5'-ATG TTC AGA GAA ATG CGG CGT AAG CG-3') and NIM5 (5'-GCT TCC TTG CCT GTC ATG TGC TC-3') directed to the *nim* genes were used [18]. Amplification reactions were performed in a volume of 50 µL containing 5 µL, 0.5 µL and 0.1 µL of DNA templates in dirPCR, imsPCR and purePCR, respectively, 1.5 mM $MgCl_2$, 5 µL of ×10 *Taq* buffer (Perkin Elmer AB, Stockholm, Sweden), 200 µM each of deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate, and deoxythymidine triphosphate (Pharmacia Biotech, Uppsala, Sweden), 1 µM of each primer (GibcoBRL, Life Technologies AB, Täby, Sweden), and 0.5 U of AmpliTaq polymerase (Perkin Elmer AB, Stockholm, Sweden). Cycling parameters were as follows: an initial denaturation at 94°C for 10 min; 30 cycles of 94°C for 30 s, 62°C for 1 min and 72°C for 1 min; and a final extension at 72°C for 10 min. The expected product was a DNA fragment with a length of 458 bp.

Agarose gel electrophoresis

The amplified products were subjected to electrophoresis in a 2% agarose gel containing ethidium bromide (0.25 mg/L) and photographed under UV illumination by using the Polaroid MP4 system. A D-15 DNA marker (NOVEX, San Diego, Ca, USA) was used as a molecular size standard.

Preparation of spiked fecal samples for PCR analysis

A stool sample was obtained from a healthy volunteer. *B. fragilis* KSB 1468/90 [10,12] was used as a *ccrA*-positive reference strain. Bacteria were suspended in PBS to achieve an optical density at 600 nm of 1.0 (approximately 2×10^9 CFU/mL). Serial 10-fold dilutions were prepared and added to preweighed stool samples to attain concentrations ranging from 10 to 10^9 CFU/g of feces. A non-spiked fecal sample (0.1 g/mL in PBS) and a pure bacterial suspension in PBS (10^8 CFU/mL) were included as negative and positive controls, respectively. The spiked fecal samples were processed by two methods (dirPCR and imsPCR) to determine the sensitivity of each assay. *B. uniformis* 2935 isolated in this study was used to spike the stool sample to determine the sensitivity of dirPCR for *nim* in this strain.

RESULTS

Resistant strains

Among the 925 fecal samples, two imipenem-resistant *B. fragilis* strains (*B. fragilis* 603 and 1619) and six metronidazole-resistant *B. fragilis* group strains (*B. distasonis* 782 and 3667, *B. fragilis* 1619, *B. thetaiotaomicron* 2877, *B. uniformis* 2935 and *B. vulgatus* 2985) were detected by the modified disk diffusion method (Table 1). *B. fragilis* 1619 was simultaneously resistant to imipenem and metronidazole. The MICs for imipenem and metronidazole of these strains were further determined by the agar dilution method (Table 1). *B. fragilis* 603 and 1619 were highly resistant to imipenem, with MICs >32 mg/L. Among the metronidazole-

Table 1 Resistant strains detected by the modified disk diffusion method, their MICs by the agar dilution method and detection of resistance genes

	Modified disk diffusion method		MICs (mg/L) Agar dilution method		Presence of resistance genes	
	Imipenem	Metronidazole	Imipenem	Metronidazole	*ccrA*	*nim*[a]
Bacteroides fragilis 603	R	S	>32	0.5	+	−
B. distasonis 782	S	R	1	0.5	−	−
B. fragilis 1619	R	R	>32	32	+	−
B. thetaiotaomicron 2877	S	R	0.5	8	−	+
B. uniformis 2935	S	R	0.25	1	−	+
B. vulgatus 2985	S	R	0.25	8	−	+
B. distasonis 3667	S	R	2	0.5	−	−

R, resistant; S, susceptible.
[a]Detected by purePCR.

resistant strains detected by the modified disk diffusion method, only one of them (*B. fragilis* 1619) was regarded as resistant by the MICs according to the NCCLS [20].

Detection of *ccrA* in *Bacteroides fragilis* strains

The metallo-β-lactamase gene *ccrA* was detected from samples 603 and 1619 by all three methods (purePCR, dirPCR and imsPCR). The amount of DNA required for optimal amplification varied with different methods and different strains. Sample 603 was used as an example (Figure 1). For dirPCR, though with varying intensity, PCR products with an expected length of 442 bp were yielded when 1 μL, 5 μL or 15 μL of DNA was included in the reaction mixture. For imsPCR, positive reactions were obtained with 0.1 μL, 1 μL, 5 μL or 10 μL of DNA. For purePCR, an intensive band at the position of 442 bp was observed with 1 μL of DNA.

Sensitivities of dirPCR and imsPCR for detection of *ccrA* from spiked fecal samples

The sensitivities of the dirPCR assay and imsPCR assay were investigated by use of spiked fecal samples. The limits for the detection of *ccrA* by dirPCR and imsPCR were ~10^5 CFU/g of feces and ~10^4–10^5 CFU/g of feces, respectively (Figures 2 and 3). For dirPCR, the lower limits of detection with 1 μL, 5 μL or 15 μL of DNA were 10^7, 10^5 and 10^6 CFU/g of feces, respectively. The most sensitive reaction was obtained with 5 μL of DNA in the reaction mixture (Figure 2). For imsPCR, the suitable amount of DNA in the reaction mixture was 0.1 μL (Figure 3).

Detection of *nim* genes in *Bacteroides fragilis* group strains

Among the six metronidazole-resistant *B. fragilis* group strains detected by the modified disk diffusion method, the *nim* genes were detected by dirPCR from samples 1619, 2877 and 2985, by imsPCR from sample 1619, and by purePCR in strains *B. thetaiotaomicron* 2877, *B. uniformis* 2935 and *B. vulgatus* 2985, but not in *B. fragilis* 1619 (Figure 4 and Table 1). The lower limit for detection of *nim* by the dirPCR assay from fecal samples spiked with *B. uniformis* 2935 was 10^8 CFU/g of feces.

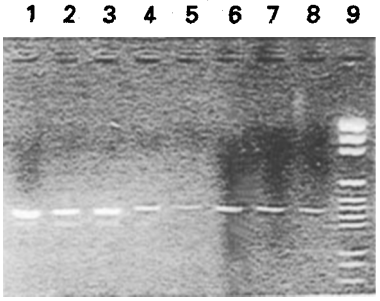

Figure 1 Agarose gel electrophoresis of PCR products from sample 603 for detection of *ccrA*. Lane 1: purePCR with 1 μL of DNA. Lanes 2–5: imsPCR with 10 μL, 5 μL, 1 μL and 0.1 μL of DNA, respectively. Lanes 6–8: dirPCR with 15 μL, 5 μL and 1 μL of DNA, respectively. Lane 9: DNA molecular size marker (D-15 DNA marker; NOVEX, San Diego, Ca, USA).

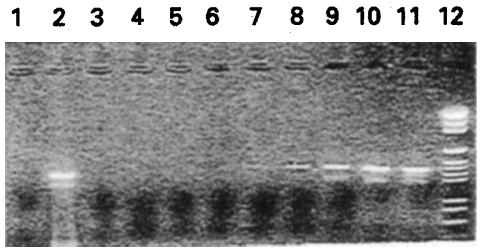

Figure 2 The dirPCR products of *ccrA* from spiked fecal samples prepared with serial dilutions of *B. fragilis* KSB 1468/90. Lane 1: negative control. Lane 2: positive control. Lanes 3–11: dilutions from 10 to 10^9 CFU/g of feces, respectively. Lane 12: DNA molecular size marker.

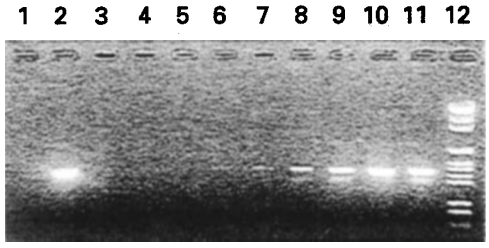

Figure 3 The imsPCR products of *ccrA* from spiked fecal samples prepared with serial dilutions of *B. fragilis* KSB 1468/90. Lane 1: negative control. Lane 2: positive control. Lanes 3–11: dilutions from 10 to 10^9 CFU/g of feces, respectively. Lane 12: DNA molecular size marker.

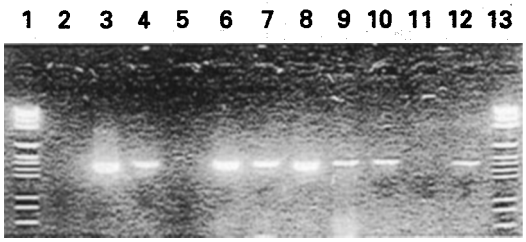

Figure 4 Agarose gel electrophoresis of PCR products for detection of *nim*. Lanes 1 and 13: DNA molecular size marker. Lane 2: negative control. Lane 3: positive control. Lane 4: sample 1619 by imsPCR. Lanes 5–8: *B. fragilis* 1619, *B. vulgatus* 2985, *B. uniformis* 2935 and *B. thetaiotaomicron* 2877 by purePCR. Lanes 9–12: samples 1619, 2985, 2935 and 2877 by dirPCR.

DISCUSSION

In this study, two strains (*B. fragilis* 603 and 1619) were considered to be resistant to imipenem and one strain (*B. fragilis* 1619) to metronidazole according to their MICs. They were isolated from fecal samples of diarrhea patients. The rates of imipenem-resistant and metronidazole-resistant *B. fragilis* group strains in diarrhea patients examined were 0.27% (2/729) and 0.14% (1/729), respectively. The incidence of resistance to imipenem or metronidazole in *B. fragilis* group strains was low in this survey, which is in accordance with other surveys [4–6]. One isolate (*B. fragilis* 1619) was found to be resistant to both imipenem and metronidazole. It was isolated from the fecal sample of a 24-year-old female patient with diarrhea. Besides *B. fragilis* 1619, *Clostridium* sp. was isolated on the BKV plate from this sample. Simultaneous resistance to imipenem and metronidazole in *B. fragilis* is unusual. *B. fragilis* KSB 1468/90 reported previously was this kind of multiresistant strain which was isolated from the blood of a patient with septicemia [10], and in a report by Turner et al in 1995 a similar strain was described [7]. Since options for the treatment of *B. fragilis* are limited [21], the appearance of multiresistant strains will give rise to a refractory problem in clinical medicine.

The modified disk diffusion method was employed to screen for resistant strains. The detected imipenem-resistant strains had high MICs (>32 mg/L) as determined by the agar dilution method. The results of these two methods were in agreement in the case of imipenem. When confirming the metronidazole resistance by the agar dilution method, only one strain (*B. fragilis* 1619) reached the breakpoint (32 mg/L) in terms of MIC, and two strains (*B. thetaiotaomicron* 2877 and *B. vulgatus* 2985) had MICs near the breakpoint (Table 1). Several factors may be responsible for the discrepancy between these two methods. The number of microorganisms in feces and the dilution of microorganisms in loose or watery stools can lead to a varying inoculum in the modified disk diffusion method. It is also known that *Bacteroides* species can be protected against the killing effect of metronidazole in the presence of *Enterococcus faecalis* [22,23]. Although *Bacteroides* strains were enriched after PYG broth incubation, the bacteria applied to the plate in this assay still comprised a mixed culture. This might be the reason why the number of resistant strains detected by the modified disk diffusion method was higher than the number of resistant strains confirmed by the agar dilution method. In any case, the modified disk diffusion method used in the present study was a practical and effective screening method when dealing with large numbers of samples.

The carbapenem-hydrolyzing metallo-β-lactamase gene (*ccrA*) was detected from samples 603 and 1619 by purePCR, dirPCR, and imsPCR. The amount of DNA required for each assay is different, which reflects the difference in the concentration of DNA obtained by different processing methods, the percentage of target DNA in the whole DNA extracted, and the copy number of the target gene. In principle, anaerobes were enriched, aerobes were inhibited and PCR inhibitors from feces were diluted after incubation in PYG broth containing kanamycin (dirPCR). Therefore, the DNA used in dirPCR was mainly nucleic acid from mixed anaerobes. In imsPCR, only *B. fragilis* strains were included, since the monoclonal antibodies (MAb C3 and MAb 4H8) used in the IMS were specific for the lipopolysaccharides of *B. fragilis* [16].

For detection of *ccrA*, PCR with spiked fecal samples (*B. fragilis* KSB 1468/90) showed that there was at most one log difference in the lower limits of detection between imsPCR (10^4–10^5 CFU/g of feces) and dirPCR (10^5 CFU/g of feces).

The *nim* gene was detected from sample 1619 by dirPCR and imsPCR, but not by purePCR. The metronidazole-resistant strain *B. fragilis* 1619 isolated from this sample did not yield amplicon by purePCR, which indicates absence of the *nim* gene or that the *nim* gene is undetectable by the employed PCR assay. One explanation of the positive results in dirPCR and imsPCR in sample 1619 might be the presence of *B. fragilis* strains other than *B. fragilis* 1619, containing the *nim* gene. For sample 2935, the *nim* gene was detected in purePCR, but not in dirPCR. Further studies using spiked fecal samples showed that the limit for detection of *nim* by dirPCR in this strain was 10^8 CFU/g of feces. Therefore, it is believed that the concentration of *B. uniformis* 2935 in the sample was lower than this limit. Since the IMS method used in this study was specific for *B. fragilis* only, the imsPCR assay was not applied to non-*fragilis* species of the *B. fragilis* group. The *nim* genes were also detected in samples 2877 and 2985 (strain 2877 and strain 2985, MIC 8 mg/L for metronidazole), by both pure PCR and dirPCR, but not in samples 603, 782 and 3667 (MIC for metronidazole 0.5 mg/L). The metronidazole susceptibilities were further determined by disk diffusion from the isolated strains. There was no inhibition zone for strains with MICs 8–32 mg/L. As we know, the breakpoint for metronidazole susceptibility is ≤4 mg/L in Spain and France, and the breakpoint for metronidazole resistance is ≥16 mg/L in Sweden. According to the present findings, regarding the presence of *nim* genes (strains with MICs ≥1 mg/L), the NCCLS breakpoint for metronidazole might be too high.

The dirPCR assay is simpler but somewhat less sensitive than the imsPCR assay for the detection of

ccrA from fecal samples. The dirPCR assay is not limited by the specificity of the monoclonal antibodies, which makes it applicable not only to *B. fragilis* species but also to other bacterial species. Both methods are free of complexity of isolation and identification of the microorganism among the complex fecal bacterial flora.

In conclusion, the incidences of imipenem-resistant and metronidazole-resistant *B. fragilis* group strains were low in the investigated diarrhea patients. Simultaneous resistance to imipenem and metronidazole is of great concern in clinical medicine. Resistance genes can be detected from fecal samples without pure bacterial isolates. This may be useful in epidemiologic studies of the distribution of resistance genes in the fecal microflora.

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